Antennal and Behavioral Responses of *Lygus lineolaris* (Palisot de Beauvois) (Heteroptera: Miridae) to Metathoracic Scent Gland Compounds'

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Abstract Hexyl and (E)-2-hexenyl butyrates and (E)-4-oxo-2-hexenal are major components of the metathoracic scent Oland (MSG) secretion and aeration samples of many plant bugs (Middae), including the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). Laboratory and field experiments were performed (1) to determine the extent to which MSG-produced compounds are detected by antennae of *L. lineolaris* males and females, and (2) to elucidate the behavioral activity associated with the antennally active MSG compounds. The three major MSG-produced compounds elicited strong antennal responses by both sexes. In Y-track olfac- tometer tests, attraction of males to virgin females was significantly reduced when a dispenser loaded with hexyl butyrate was placed with the virgin females. Hexyl butyrate tested alone against a blank control significantly repelled males. In the field, ternary and partial binary com- binations of these three EAD-active compounds failed to attract either sex, whereas virgin females attracted a significant number of males. However, addition of hexyl butyrate and/or (E)-2-hexenyl butyrate to virgin female-baited traps significantly reduced the number of males caught compared with the level of the blank control traps. This might be due either to a negative effect on pheromone release by the females or direct repellency of males, or both. These MSG compounds may be useful for mating disruption and other pest management tactics against economically important plant bugs.

4(ey Words tarnished plant bug, cotton, samiochemicat, aiic)mone, attracbon, Tepeilent, mat- ing disruption, pest management, electroantennogram

Nearly 10,000 species of mirid plant bugs (Heteroptera: Miridae) have been described worldwide, many of which are severe crop pests requiring insecticidal treatment, especially lygus bugs (Schuh and Slater 1995, Wheeler 2001). The problem with lygus bugs in cotton has increased in recent years because of their immunity to the Bt-endotoxin in transgenic cotton (Nordlund 2000) and resistance to pesticides (Snodgrass and Scott 2000). Despite their pest status, sampling and monitoring

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methods for mirids are relatively primitive, consisting mainly of beating-tray and sweep-net sampling.

Sex pheromone-baited traps are an important tool for detecting and monitoring many agricultural pest insects; the development of this technique would facilitate the scouting and timing of control measures for economically important plant bugs. Mat- ing disruption (McBrien et al. 1996, McBrien et al. 1997, Kakizaki 2004) and mass trapping are other potential uses of sex pheromones to directly control plant bug pests. Despite progress in the identification of mirid pheromones (Smith et al. 1991, Millar et al. 1997, Millar and Rice 1998, Kakizaki and Sugie 2001, Downham et al. 2002, Zhang and Aldrich 2003a, lnnocenzi et al. 2005), the sex pheromones of *Lygus lineolaris* (Palisot de Beauvois) (the tarnished plant bug) and *L. hesperus* Knight (the western plant bug), remain unknown (McLaughlin et al. 1998).

What is known of mirid sex pheromones indicates that they are produced in the metathoracic scent gland (MSG) of females (e.g. Zhang and Aldrich 2003a), but data presented by Graham (1 988) indicated that in *L. hesperusvolatiles* from the ovipositor are involved in mate attraction. Males and females also abundantly produce MSG compounds (usually esters, such as hexyl butyrate and/or (E)-2-hexenyl butyrate) that seem not to be part of the sex pheromone system (Aldrich 1988, Aldrich et al. 1988, Ho and Millar 2002, Zhang and Aldrich 2003a). The toxicity of mirid MSG compounds for predators has, with one notable exception (Staples et al. 2002), rarely been investigated in detail, although Ho (2000) also found that MSG esters of L. *hesperus* act as an alarm pheromone. In most cases, however, chemical identifica- tions of mirid exocrine compounds have not been accompanied by bioassays **of** their behavioral effects, so the function of MSG compounds remains largely speculative.

The goals of the work described herein were (1) to determine the extent to which MSG-produced compounds are detected by antennae of *L. lineolaris* males and females, and (2) to elucidate the behavioral activity associated with the antennally active MSG compounds.

Material and **Methods**

Adult insects and preparation of extracts. Virgin females and males of 7-10- day-old *L. lineolatis* adults used for volatile sampling (both sexes) and field trapping (females only) were shipped by overnight delivery from the USDA-ARS Southern Insect Management Research Unit, Stoneville, MS, to the Beltsville laboratory. MSGs were excised from CO,-anesthetized bugs submerged in tap water, and the glands were extracted individually in 50 VL of methyl-tert-butyl ether. Aeration samples were collected from groups of either 1 0 males or females in 1 -L 4-necked glass chambers, each containing 5 fresh green beans and a water bottle. Air was drawn by vacuum (1.0 Umin for 24 h) into the chamber through a column of 6-14 mesh activated charcoal, and out of the chamber through two Super ^{QTM} traps (200 mg each, 50-80 mesh, Alitech Associates, Inc., Deerfield, IL). Each Super ^{QTM} trap was rinsed with 2 ml of CH_2Cl_2, concentrated to 200 pL with a stream of N_2, and stored at -20'C until use. One to 5 pL of an internal standard (geranyl acetate; 10 pg/UL) was added to some of the gland and aeration extracts for quantification.

Gas chromatographic-electroantennographic detection (GC-EAD) analysis. MSG and aeration extracts were analyzed using an HP 6,890 GC equipped with a DB-WaxETR column (30 *m* x 0.25 mm x 0.25 lim; splitiess mode; J&W Scientific Inc., Folsom, CA) programmed from 50'C for the first 2 min to 2400C at 1 O'C/rnin isother-

mally for 10 min using helium as carrier. A 1:1 effluent splitter allowed simultaneous flame ionization detection (FID) and EAD analysis (Zhang and Aldrich 2003b). The male antennae preparation was positioned in a humidified air-stream (0.5 m/s). A glass capillary indifferent electrode was filled with Beadle-Ephrussi Ringer solution (Zhang et al. 2000), grounded via a silver wire, and inserted into the open side of the severed mirid head. A similar recording electrode connected to a high-impedance DC amplifier with automatic baseline drift compensation was inserted over the distal ends of both antennae (the tip of each antenna was cut off). The antennae signals were stored and analyzed on a PC equipped with a serial IDAG interface box and the program EAD version 2.5 (Syntech, Hilversum, The Netherlands).

Compound identification. The following authentic standards were purchased: 1-hexanol, (E)-2-hexenol, (E)-2-hexenal (Aldrich Chemical Co., Milwaukee, WI); hexyl butyrate, (E)-2-hexenyl butyrate, geranyl acetate (Bedoukian Research Inc., Danbury, CT). (E)-4-oxo-2-Hexenal was synthesized as previously described (Ward and VanDorp 1969, but see: Moreira and Millar 2005). Both MSG and aeration samples were analyzed by GC-mass spectrometry (MS) on an HP 6890 GC series coupled with an HP 5973 Mass Selective Detector using the same type of GC column and conditions as described above. Compounds were identified by comparison of retention times with those of authentic standards and with mass spectra of standards.

Y-track walking olfactometer bioassay. Y-track olfactometer assays were con- ducted as described by Groot et al. (2001), with slight modification. The Y-track olfactometer was placed in a small insect net-cage box (35 **x** 40 x 45 cm) under a dim light, which was kept in a black cubical (1.1 x 1.2 x 2.3 m). One hour before each experiment, 5 virgin females (approx. 7 d old) were placed in a Mason glass jar (height 7 cm; diameter 8 cm) with three 4-cm pieces of fresh green beans; the control glass jar contained only green beans. Both jars were placed outside the cubical under ambient room temperature and fight conditons, and charcoal-fiftered air was passed through each jar (I Umin) to the arms of the Y-track. Treatments included 5 virgin females versus a blank control, 5 virgin females + a hexyl butyrate dispenser (a closed polyethylene (PE) vial; see Table 1) in the same container versus a blank control, and a hexyl butyrate dispenser alone versus a blank control. All experiments were repeated 3 times, with 10-20 males tested individually in each experiment. Individual males were released at the base of the Y-track and allowed to choose which way to walk. After testing 5 males, the glass jars and tubing systems were shifted between the 2 arms to minimize the potential positional effects.

Field trapping. Field experiments were conducted at 2 sites during the summers of 2002 and 2003, using Jackson delta traps with removable sticky inserts (Agrisense, Fresno, CA) (Zhang and Aldrich 2004). Site 1 (2002 test) was an 8.5-ha alfalfa field at the IJSDA Beltsville Agricultural Research Center (BARC) West, Prince George's Co., MD. Site 2 (for 2003 tests) was a 2-ha meadow, about 5 km east of site 1, surrounded by a mixture of deciduous and coniferous trees near Entomology Road, BARC-East. Traps were hung on metal posts 50-60 cm above ground with -10 m between traps within each trap line, and -15 m between trap lines. For each experi- ment, two sets of traps were deployed in a Latin-square design with their initial trap positions within each set randomized and rearranged after each replicate (when -_1 *Lygus* bug was caught in the best traps) (Byers 1991) to minimize positional effects. Sticky inserts with captured bugs were removed and replaced with fresh inserts after each replicate.

Experiment I (1 7 July-6 August 2002) was conducted at site 1 (alfalfa) to test the

Table 1. Chemicals, release rates and dispensers used in the behavioral experiments

Chemical	Release rate (mg/d)*	Dispenser	Y-track bioassay	Field experiment no.			
				1	2	3	4
Hexyl butyrate (HB)	4.2	100 µL in a closed PE-vial**	√	√	√		
(*E*)-2-Hexenyl butyrate (E2HB)	1.4	100 µL in a closed PE-vial		√	√		
Combination of HB/E2HB (1:1)	ND	0.02-200 mg diluted in 2 ml octane in glass vial wick dispensers				√	
Combination of HB/E2HB/E-4-oxo-2-hexenal (2:2:1)	ND	0.02-200 mg diluted in 2 ml octane in glass vial wick dispensers†					√

* Measured in a chemical ventilation hood at 20-23°C for a week. ND: not determined.

** PE-vial: polyethylene vial (#130 Capsule, Size 00; BEEM, Inc., Bronx, NY).

† 2-ml glass vial with 2 mm hole on the lid; wick = 2mm OD Teflon tubing filled with cotton rope; 3.5 cm long.

effect of the two major MSG-produced compounds (hexyl butyrate and (E)-2-hexenyl butyrate) on attraction of tarnished plant bug males to virgin females. Traps were baited with three 7-d-old virgin females in 30-mi plastic cups having perforations in the bottom, a screen lid, and two 3-cm-long pieces of green bean. Each cup containing females was suspended from the roof of the trap, and virgin females and beans were renewed after the **3rd** day of exposure in the field. Chemical dispensers consisted of PE vials loaded with either hexyl butyrate, (E)-2-hexenyl butyrate or both (Table 1), which were placed in the plastic cups with virgin females. Experiment 2 (16-23 June 2003) was conducted at site 2, with the same treatments as in Experiment 1, but the PE vials containing hexyl butyrate and/or (E)-2-hexenyl butyrate were suspended outside, but adjacent to the cage of virgin females. Experiment 3 (16-23 June 2003, at site 2) tested the potential dose-responses of TPBs to the combination of,hexyl butyrate and (E)-2-hexenyl butyrate (1:1) using wick-dispensers (Table 1). Butyrates were dissolved in 2 ml of octane at different concentrations (ranging from 0-1 0% by volume) and released from wick-dispensers constructed of 2-ml glass vials with a 2 mm hole in the lid containing a wick made of a 2 mm piece of Teflon tubing and a 3.5-cm-long piece of cotton rope (Schlyter et al. 2001). A trap baited with virgin females, as in experiments 1 and 2, was included as positive control. Experiment 4 (24-30 June 2003, at site 2) tested a combination of all three EAD active compounds (hexyl butyrate, (E)-2-hexenyl butyrate and (E)-4-oxo-2-hexenal) at a ratio of 2.2:1, again ranging from 0-1 0%. **All** chemicals, release devices, and release rates are listed in Table 1.

Statistics. Preferences for odor sources or blank controls in Y-track experiments were determined by a 2-tailed binomial test using the pooled data (sum). Due to the strong heterogeneity of variances among treatments, field-trapping data were ana- lyzed using the nonparametric Kruskal-Wallis ANOVA on rank test, followed by the Student-Newman-Keuis all pairwise comparison with separate means (Zar 1984).

Results

GC-EAD analysis. Antennae of *L. lineolaris* adults consistently responded to three major components, hexyl butyrate, (E)-2-hexenyl butyrate and (E)-4-oxo-2-hexenal from MSG extracts of both females and males with no obvious differences in EAD responses between sexes (Fig. 1 A; 1 B; EAD of females not shown). Similar Fl 0 and EAD responses also were found for the aeration samples of the tarnished plant bug (Fig. 2A, B). GC internal standard quantifications suggest that female tarnished plant
bugs produce and release more MSG volatile compounds (43.2 ± 8.8 pg/gland; n 3; and 2.0 ± 0.7 pg/bug/day; n = 3) than do males (33.0 ± 9.3 pg/gland; n = 3; 0.9
0.3 pg/bug/day; n = 3). The relative ratio of hexyl butyrate and (E)-2-hexenyl butyrate varied signif icantly between the sexes, with an almost 1: 1 ratio in females and a I 5: 1 ratio in males (Table 2). The identity of these antennally active MSG compounds was confirmed by GC retention time comparisons to synthetics and GC-MS analysis (data not shown).

Y-track walking bioassays. In the Y-track olfactometer assays, significantly more tarnished plant bug males walked toward the virgin female treatment arm than to the control ($P < 0.001$; Fig. 3); however, when a PE-dispenser loaded with 50 mg of hexyl butyrate was placed with virgin females in the jar, significantly fewer males walked to the female treatment side (Fig. 3). Further tests with a hexyl butyrate dispenser alone

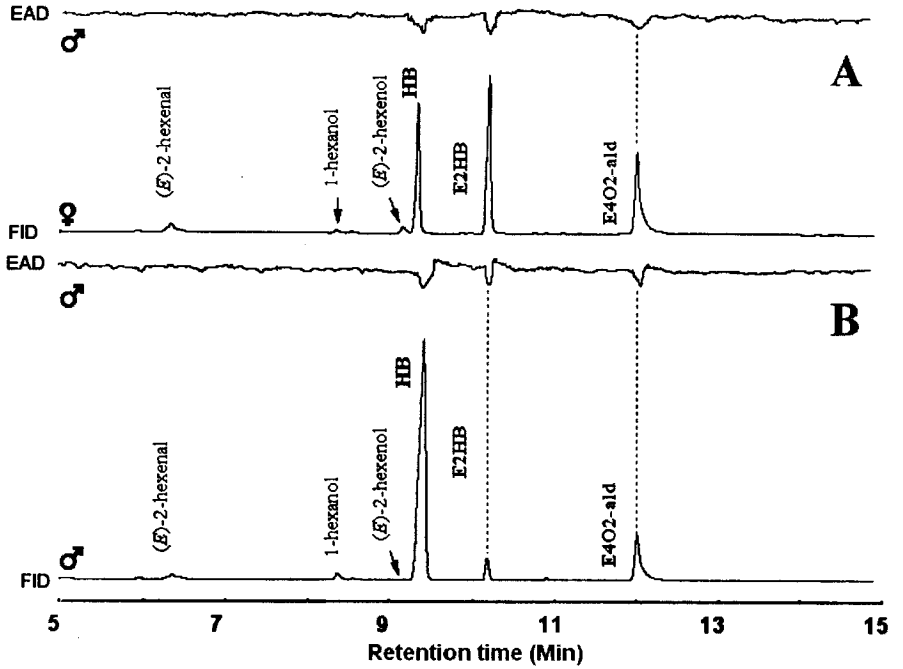

Fig. 1. GC-EAD responses of *Lygus lineolaris* male antennae to metathoracic scent gland (SMG) extracts of males (A) and females (B). HB = hexyl butyrate; E2HB = (*E*)-2-hexenyl butyrate; E4O2-ald = (*E*)-4-oxo-2-hexenal.

in the jar versus a blank control indicated that significantly more males walked toward the control than toward the hexyl butyrate dispenser side (Fig. 3).

Field trapping. In Experiment 1, traps baited with 3 virgin females alone caught significantly more males than did blank control traps (Fig. 4A). Addition of hexyl butyrate, (*E*)-2-hexenyl butyrate or their combination to the virgin female-baited traps (inside the female cages) significantly reduced the number of males caught to the level of the blank control (Fig. 4A). No synergistic effect between hexyl butyrate and (*E*)-2-hexenyl butyrate on reduction of trap catch was detected. A similar response pattern also was found in Experiment 2, where the dispensers of hexyl butyrate, (*E*)-2-hexenyl butyrate, or their combination, were placed outside cages containing females (Fig. 4B). In Experiment 3, a total of 35 males were captured, mainly in the female-baited traps. The combination of hexyl butyrate and (*E*)-2-hexenyl butyrate released from the glass vial wick dispensers was inactive at the concentration range of 0-10% (Fig. 5A). An identical result was found in Experiment 4, where a mixture of all the three EAD-active compounds was tested instead of a combination of the two esters (Fig. 5B).

Discussion

Most adult true bugs (Hemiptera: Heteroptera) have a massive metathoracic scent gland (MSG) from which they can eject noxious compounds to blunt the attack of

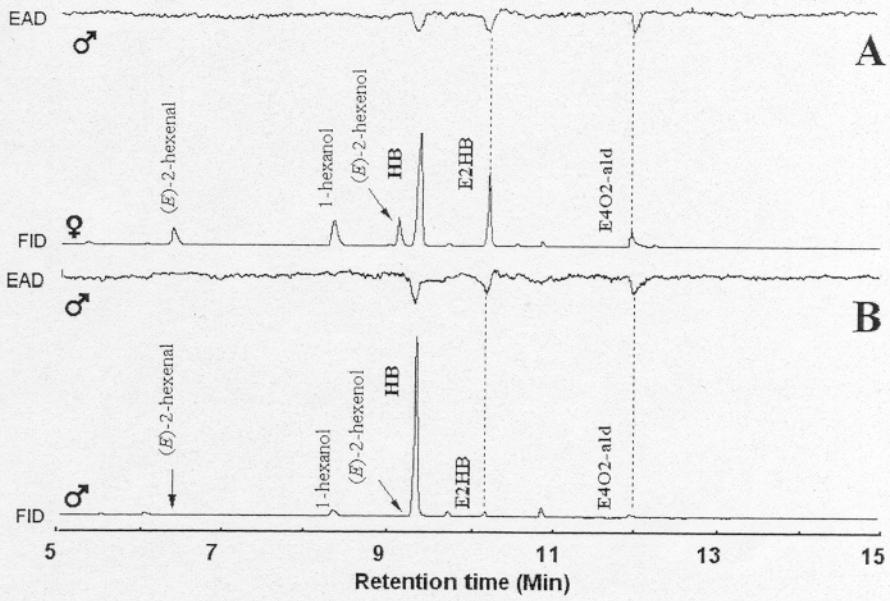

Fig. 2. GC-EAD responses of *Lygus lineolaris* male antennae to aeration samples of (A) virgin females and (B) virgin males.

Table 2. Relative amounts (%; means ± SE) of the major volatiles produced and released by virgin adults of *Lygus lineolaris*

Compounds	MSG extracts		Aerations	
	Females (N = 3)	Males (N = 3)	Females (N = 5)	Males (N = 4)
(*E*)-2-hexenal	3.9 ± 0.7	2.3 ± 0.3	5.1 ± 0.9	1.7 ± 0.9
1-hexanol	4.2 ± 3.2	2.3 ± 0.2	7.8 ± 1.5	5.7 ± 0.9
(*E*)-2-hexenol	3.0 ± 0.9	0.1 ± 0.0	7.9 ± 0.8	2.1 ± 0.5
Hexyl butyrate	34.5 ± 3.1	72.8 ± 4.8	45.4 ± 3.1	82.5 ± 3.7
(*E*)-2-hexenyl butyrate	35.8 ± 1.8	4.6 ± 0.6	23.9 ± 3.2	5.7 ± 1.6
(*E*)-4-oxo-2-hexenal	18.6 ± 5.5	17.9 ± 4.2	9.9 ± 1.3	2.4 ± 1.1

predators (Aldrich 1988). However, in many bugs these defensive glands are compartmentalized such that they can also produce sex or aggregation pheromones from the same openings used to emit allomones (Smith et al. 1991, Aldrich 1994,1996, Millar et al. 1997, Millar and Rice 1998, McBrien and Millar 1999, Zhang and Aldrich 2003a). Lygus bugs in particular, and mirids in general, are rather atypical among

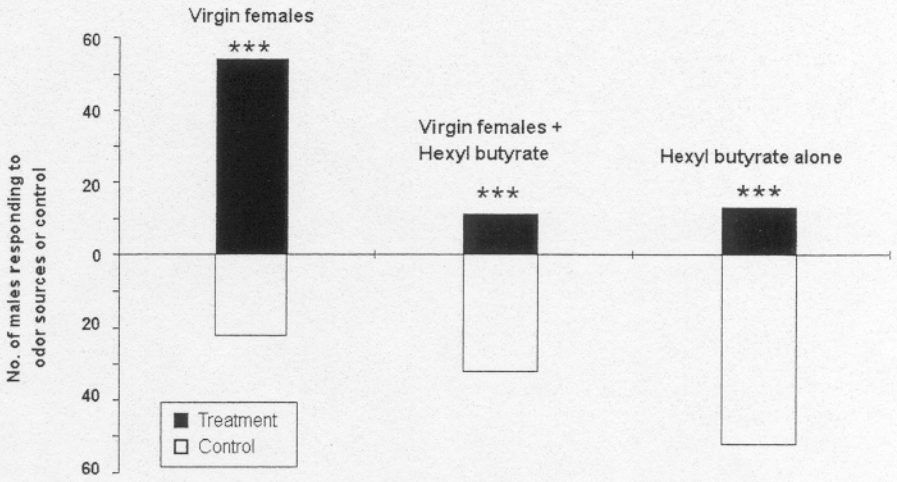

Fig. 3. Y-track olfactometer walking bioassays of *Lygus lineolaris* males. Preferences for odor sources were determined by using the two-sided binomial test; *** $P < 0.001$.

heteropterans in usually, but not always (Staples et al. 2002), producing MSG secretions that consist mainly of esters rather than aldehydes characteristic of other bugs (Aldrich 1988).

Our finding that the major components of *L. lineolaris* MSG secretion are hexyl butyrate, *(E)*-2-hexenyl butyrate and *(E)*-4-oxo-2-hexenal, with the expression of the butyrates being sexually dimorphic, agrees with past analyses of the MSG secretion for this important pest (Blumenthal 1978, Gueldner and Parrott 1978, Aldrich et al. 1988, Wardle et al. 2003). We also found that antennae of both sexes of *L. lineolaris* are most sensitive to the major components of their corresponding MSG extracts, hexyl butyrate, (*E*)-2-hexenyl butyrates and (*E*)-4-oxo-2-hexenal, again agreeing with earlier experimentation (Chinta et al. 1994). Other mirids also exhibit strong antennal responses to these butyrates and to (*E*)-4-oxo-2-hexenal, including *Trigonotylus caelestialium* (Kirkaldy) (Kakizaki and Sugie 2001), *L. rugulipennis* Poppius (Innocenzi et al. 2004), *L. hesperus* (Ho and Millar 2002), and *Lygocoris pabulinus* (L.) (Drijfhout et al. 2002). In the latter two species, the antennae of females showed either no responses or significantly less EAD activity to the butyrates or (*E*)-4-oxo-2-hexenal than did the antennae of males (Ho and Millar 2002), and this effect also was observed in *L. lineolaris* (Chinta et al. 1994). However, our GC-EAD analysis of *L. lineolaris* failed to detect sexually biased responses.

In *Lygus* spp. and some other mirids, hexyl and/or *(E)*-2-hexenyl butyrates counteract male attraction, but precisely how this effect is accomplished is not clear. Blumenthal (1978) showed that hexyl butyrate inhibited the attractiveness of caged *L. lineolaris* females in the field. Wardle et al. (2003) confirmed these results for *L. lineolaris*, and conducted field-cage experiments, suggesting that avoidance of plants due to hexyl butyrate treatment was so ephemeral as to be impractical as a control measure. For the green capsid bug, *L. pabulinus*, Groot et al. (2001) found

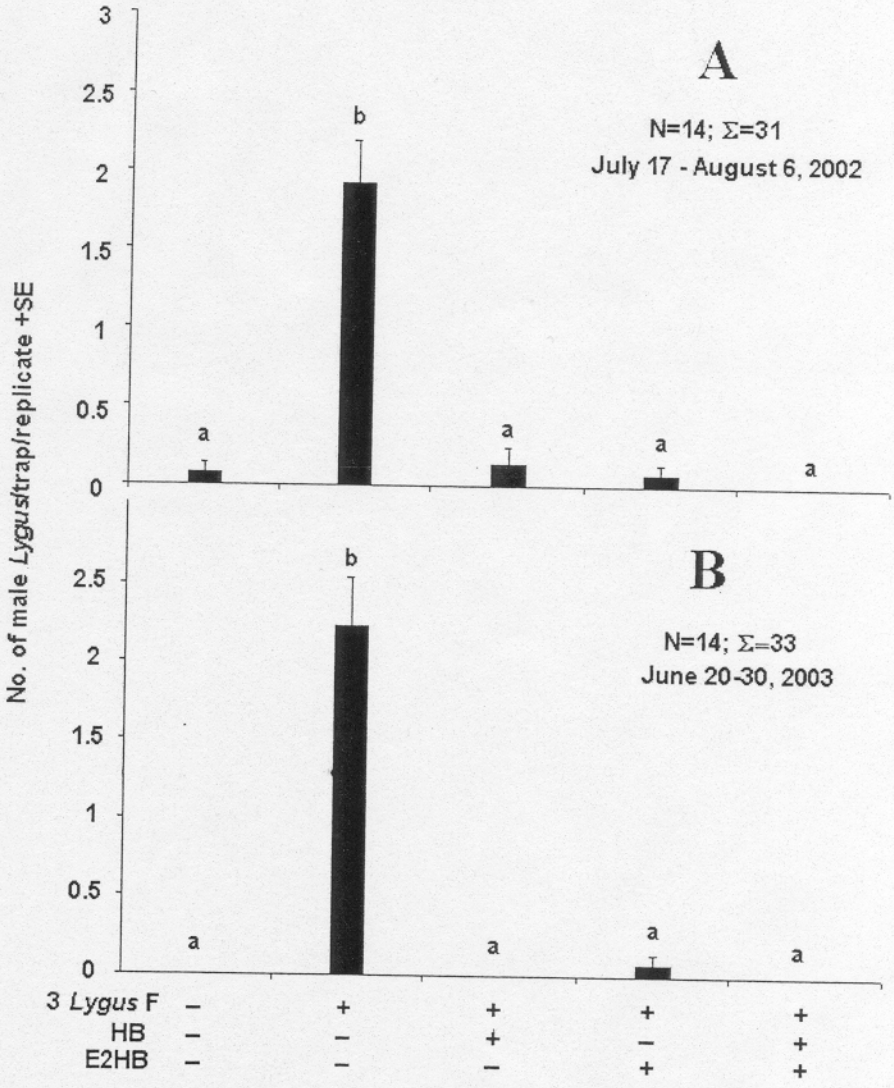

Fig. 4. Captures of *Lygus lineolaris* males in traps baited with 3-virgin females, plus hexyl butyrate (HB) or (*E*)-2-hexenyl butyrate (E2HB), or their combination. A. Experiment 1; PE-vial dispensers placed together with virgin females in the cage; alfalfa field. B. Experiment 2, PE-vials placed outside the virgin female cage; meadow field; Bars within each experiment followed by the same letter are not significantly different ($P > 0.05$), Kruskal-Wallis ANOVA on ranks, followed by the Student-Newman-Keuls all pair-wise comparison.

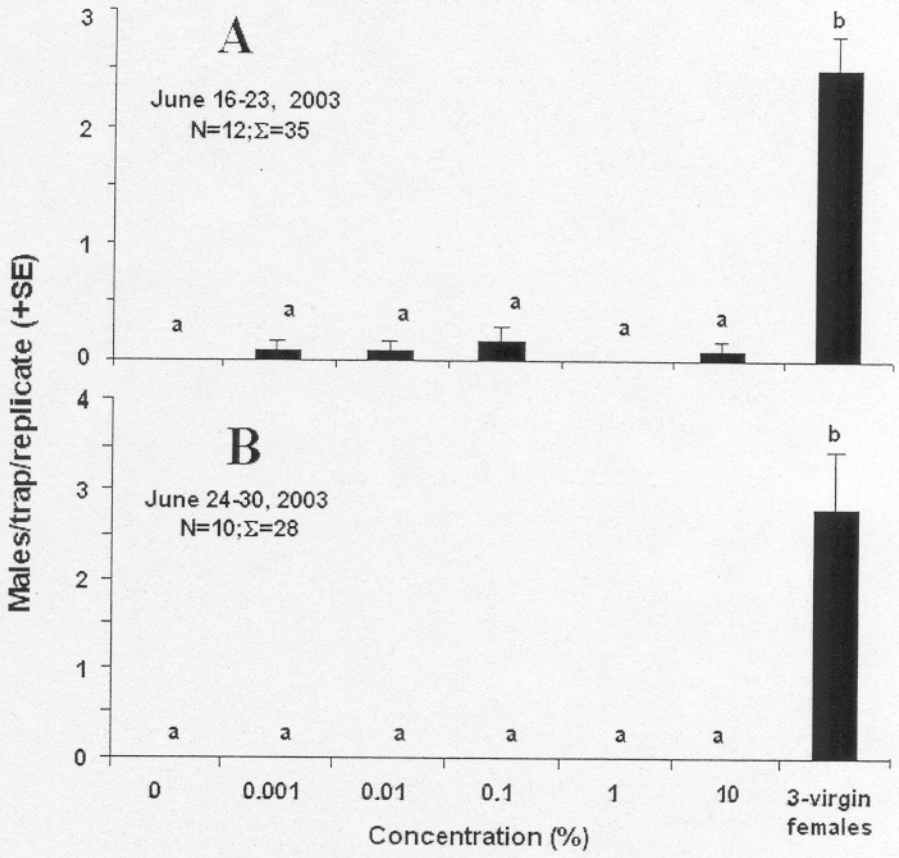

Fig. 5. Captures of *Lygus lineolaris* males in traps baited with different doses of (A) a combination of hexyl butyrate and (*E*)-2-hexenyl butyrate (at 1:1; experiment 3); (B) a combination of hexyl butyrate, (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal (at 1:1:0.52:2:1; experiment 4). Bars within each experiment followed by the same letter are not significantly different ($P > 0.05$), Kruskal-Wallis ANOVA on ranks, followed by the Student-Newman-Keuls all pair-wise comparison.

that hexyl butyrate from the MSG secretion strongly inhibited sex pheromone release in females but did not inhibit pheromone attraction of males to virgin females. Because the hexyl butyrate-mediated avoidance experiments of Wardle et al. (2003) did not include female tarnished plant bugs on the treated plants, the antipheromone aspect of hexyl butyrate was not assessed. Thus, the suppression of male attraction to virgin *L. lineolaris* females that we demonstrated in the field and in the laboratory using synthetic hexyl butyrate, (*E*)-2-hexenyl butyrate and their combination may be due to inhibition of sex pheromone release by females. Nevertheless, the direct repulsion of tarnished plant bug males by butyrates cannot be ruled out based on our

data; when we tested hexyl butyrate alone against the blank control in the Y-track offactometer, a significant repellent effect on males of *L. lineolaris* was detected. In field-trapping experiments involving another mirid *(Phytocoris difficilis* Knight) for which there is an effective synthetic sex pheromone, both hexyl butyrate and (E)-2- hexenyl butyrate completely suppressed the captures of *Phytocoris* males in traps baited with synthetic pheromone (Zhang and Aldrich 2003b).

In summary, our results confirm that tarnished plant bug females produce a sex pheromone attractive to conspecific males (see also: Wardle and Borden 2003), and verify that hexyl and (E)-2-hexenyl butyrates, at physiologically relevant doses (Zhang and Aldrich 2003a), interfere with the sex pheromone. One possible explanation is that males or females, or both, discharge hexyl and (E)-2-hexenyl butyrates from their MSG during or after mating to prevent other males from further attempts to mate. It remains unclear whether the antipheromone effect of butyrate esters is achieved directly by nullifying the perception of the female-produced sex pheromone or indi- rectly by shutting down the production or release of pheromone by females.

Interestingly, (E)-2-hexenyl butyrate was one of the major induced volatiles from cofton plants fed upon by corn earworm caterpillars (McCall et al. 1994). *Lygus hesperus* salivary gland extracts were capable of inducing emission of the same volatile bland from cofton and corn as measured for plants infested by chewing insects or treated with volicitin, an elicitor isolated from caterpillar regurgitant (Rod- riguez-Saona et al. 2002). Feeding by *L. lineolaris* on cofton reportedly induced the release of volatiles, although butyrates were not among the induced volatiles detected (Williams et al. 2005). Even so, butyrates might be part of a general plant chemical defense against plant bugs similar to the production of aphid alarm pheromones from wild potato plants (Gibson and Pickeft 1983).

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